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(54) Title: GRASS ENDOPHYTES

(57) Abstract: The present invention relates to a combination of a Neotyphodium species endophyte or endophyte culture in a symbiotic association with a host grass which association does not cause symptoms of toxicosis in animals through exclusion of ergovaline but, due to the inclusion of alkaloids including agroclavine, setoclavine or isosetoclavine, retains the ability to resist abiotic stresses and protect the grass from pests.

GRASS ENDOPHYTES

TECHNICAL FIELD

This invention relates to fungal endophytes and combinations of endophytes with grass plants. More particularly the invention relates to endophytes which form combinations with tall fescue (*Festuca arundinacea*) and some other related grasses. Even more particularly the invention relates to combinations having reduced toxicity to grazing livestock as compared to cultivars of endophyte/tall fescue combinations in common use.

BACKGROUND ART

Fungal endophytes of the genus *Neotyphodium* (formerly *Acremonium*) infect a number of temperate climate Pooideae grasses. The *Neotyphodium* endophytes can produce alkaloids which are considered to confer degrees of pest and possibly disease protection upon the plants in which they naturally occur (Rowan and Latch, 1994; Blank and Gwinn, 1992). The *Neotyphodium* endophytes are vertically transmitted through the seed of the grasses and no natural horizontal transmission has been established (Leuchtmann,1997).

Many of the predominating natural endophyte infections of improved grass cultivars used for pastoral agriculture production also cause significant animal disorders, for example tall fescue toxicoses (Stuedemann and Hoveland, 1988) and ryegrass staggers (Fletcher et al., 1999). These may be complex toxic reactions by animals to alkaloids produced under a range of plant growth conditions. Significant economic loss within pastoral agriculture systems can occur due to such animal toxicoses. On the other hand presence of at least some endophytes may be essential for the competitive persistence of the chosen grass in a pasture (Elberson and West, 1996, Fletcher and Easton, 2000).

Grass lines can be artificially infected with selected endophytes. Axenic cultures of endophytes can be used to infect grass seedlings, grown initially under sterile conditions (Latch and Christensen, 1985), which are then selected for desirable qualities, and multiplied for commercial use. Three significant examples of this technology have been developed by the Grasslands division of AgResearch Ltd: GREENSTONE™ tetraploid hybrid ryegrass with ENDOSAFE™ endophyte (Tapper

and Latch, 1999, NZ Patent 233083); various perennial and hybrid ryegrasses with AR1 endophyte (Fletcher and Easton, 2000); and tall fescue cultivars with MaxQ™ endophyte (Bouton, 2000; Bouton et al., 2002, US Patent 6,111,170).

Fescue toxicosis

Fescue toxicosis has been associated with the natural infection of tall fescue by common strains of *Neotyphodium coenophialum*. These strains typically produce the ergopeptine alkaloid, ergovaline, which is of a class of ergopeptines known to be toxic to mammals. Ergovaline is considered to be the primary cause of fescue toxicity. Other compounds, notably other ergoline and ergolene compounds, for example lysergic acid, possibly add to the syndrome (Oliver, 1997; Gadberry et al., 1997; Hill et al., 2001).

The ergovaline levels tend to be higher in leaf sheath and heads of tall fescue than in leaf blade and undergo seasonal variation (Rottinghaus et al., 1991). There is very little ergovaline in roots. Typically a concentration of ergovaline in herbage or herbage products such as hay, straw, seed or silage of greater than an average of 0.4 ppm of dry matter has been considered a risk of causing fescue toxicosis (Tor-Agbidye et al., 2001) especially when combined with climatic conditions exacerbating fescue toxicosis symptoms.

Other compounds recognised as plant defence mechanisms – peramine, lolines

Peramine is produced in endophyte-infected grass (Rowan et al., 1986) and probably mobilised within the plant. It is a potent feeding deterrent for a range of insects, e.g. Argentine stem weevil (*Listronotus bonariensis*), (Rowan and Latch 1994) and a significant factor for protecting endophyte-infected grasses from insect pest predation.

Lolines (N-formylloline, N-acetylloline, N-acetylnorloline and other closely related compounds) are produced by some *Neotyphodium* endophytes including *N. coenophialum* typical of tall fescue. These compounds in appropriate endophyte-infected grasses have properties of deterring or resisting a number of insects, notably sucking insects, for example, *Rhopalosiphum padi* (Seigel et al., 1990).

Protective effects in tall fescue pastures – persistence under stress conditions

Endophyte infection has been associated with enhanced persistence of tall fescue plants under water deficit or drought conditions. Whether this effect is due to better resistance of biotic stress factors expressed in water deficit situations; general better health of endophyte-infected tall fescue plants (particularly of root systems); or due to specific differential physiological responses of the endophyte-infected plants to water stress; is not clearly evident. However, the overall effect is enhanced resistance to water deficit.

Tremorgens

Some *Neotyphodium* endophytes, notably those of evolutionary derivation from strains of *Epichloë festucae* such as *N. lolii*, produce potent tremorgens which are toxic to grazing animals. To ensure such tremorgens are not produced by an endophyte artificially introduced into forage tall fescue or ryegrass, the presence of the known potent tremorgens typical of endophytes, that is the lolitrems, is tested for. Also tremorgenic activity in grazing test animals is looked for.

It is an object of the present invention to provide an endophyte which can produce ergovaline and some ergoline and ergolene compounds at the base of the tall fescue leaf sheaths and in the crown of the plant but only in a manner such that the usual concentration in herbage as generally consumed by grazing animals in common farming practice is less than a practical threshold toxicity level.

For the purposes of this specification "crown" is defined as that area of a grass plant which is generally less than 2 cm above soil level and excludes the roots of the grass, but includes the base of tillers and lateral meristem growing points for new vegetative tillers.

It is a further object of the invention to provide an endophyte which can produce lolines in amounts which are considered to be partially or substantially effective in deterring some insect pests from feeding on plants.

It is a further object of the invention to provide an endophyte which does not produce detectable levels of lolitrems and are not observably tremorgenic.

It is a still further object of the present invention to address the foregoing problems or at least to provide the public with a useful choice.

All references, including any patents or patent applications cited in this specification are hereby incorporated by reference. No admission is made that any reference constitutes prior art. The discussion of the references states what their authors assert, and the applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of prior art publications are referred to herein, this reference does not constitute an admission that any of these documents form part of the common general knowledge in the art, in New Zealand or in any other country.

It is acknowledged that the term 'comprise' may, under varying jurisdictions, be attributed with either an exclusive or an inclusive meaning. For the purpose of this specification, and unless otherwise noted, the term 'comprise' shall have an inclusive meaning - i.e. that it will be taken to mean an inclusion of not only the listed components it directly references, but also other non-specified components or elements. This rationale will also be used when the term 'comprised' or 'comprising' is used in relation to one or more steps in a method or process.

Further aspects and advantages of the present invention will become apparent from the ensuing description which is given by way of example only.

DISCLOSURE OF INVENTION

According to one aspect of the present invention there is provided an endophyte of *Neotyphodium coenophialum* species, selected from the group consisting of: AR512; AR513; AR514; AR517; AR521; AR522; AR524; AR525; AR535; AR539; and combinations thereof; AR512; AR513; AR514; AR517; AR521; AR522; AR524; AR525; AR535, AR539 being cultures deposited on 2 October 2002 at the Australian Government Analytical Laboratories (AGAL) with accession numbers: NM02/31935; NM02/31936; NM02/31937; NM02/31938; NM02/31939; NM02/31940; NM02/31941; NM02/31942; NM02/31943; NM02/31944;

characterised in that, in combination with a host grass, said endophyte does not cause symptoms of toxicosis in animals;

and further characterised in that the endophyte retains sufficient levels of at

least two alkaloids selected from the group consisting of: agroclavine; setoclavine; isosetoclavine; and combinations thereof, that protect the host grass from pests or abiotic stresses or both;

and further characterised in that the host grass is artificially inoculated with the endophyte.

According to a further aspect of the present invention there is provided an endophyte culture of *Neotyphodium coenophialum* species, selected from the group consisting of: AR512; AR513; AR514; AR517; AR521; AR522; AR524; AR525; AR535; AR539; and combinations thereof; AR512; AR513; AR514; AR517; AR521; AR522; AR524; AR525; AR535, AR539 being cultures deposited on 2 October 2002 at the Australian Government Analytical Laboratories (AGAL) with accession numbers: NM02/31935; NM02/31936; NM02/31937; NM02/31938; NM02/31939; NM02/31940; NM02/31941; NM02/31942; NM02/31943; NM02/31944;characterised in that, in combination with a host grass, said endophyte culture does not cause symptoms of toxicosis in animals;

and further characterised in that the endophyte culture retains sufficient levels of at least two alkaloids selected from the group consisting of: agroclavine; setoclavine; isosetoclavine; and combinations thereof, that protect the host grass from pests or abiotic stresses or both;

and further characterised in that the host grass is artificially inoculated with the endophyte culture.

Preferably, the toxicosis which is avoided is fescue toxicosis. Most preferably the toxicosis is caused by an ergovaline toxin.

Preferably, the level of ergovaline in the present invention is less than 0.4 ppm in dry matter in herbage consumed by grazing animals. More preferably, the level of ergovaline is less than 0.4 ppm in dry matter in herbage, other than the crown of the host grass plant, consumed by grazing animals.

Preferably, the abiotic stress is a water deficit.

Preferably, the endophyte culture, if used, is an axenic culture.

Preferably, the endophyte or endophyte culture produces less than 0.2 ppm ergovaline in dry matter of whole herbage when infected into host grass.

According to a further aspect of the present invention there is provided a combination of the endophyte as described above, and a host grass.

According to another aspect of the present invention there is provided a combination of the endophyte culture as described above, and a host grass.

Preferably, the combination, substantially as described above, is achieved by modification of host grass infected with the endophyte or endophyte culture by methods selected from the group consisting of: breeding; crossing; hybridisation; genetic modification; and combinations thereof.

Preferably, the host grass used in the combination described above is selected from the group consisting of: tall fescue grass cultivar, ryegrass cultivar, meadow fescue cultivar, and combinations thereof.

According to a further aspect of the present invention the host grass is a Pooideae grass.

According to a further aspect of the present invention there is provided a combination of endophyte or endophyte culture, as described above, and a host grass wherein the combination produces isosetoclavine and setoclavine at a rate of greater than 0.5 ppm each of dry matter in the host grass plant crowns. Preferably also, the combination produces less than 0.2 ppm of dry matter of ergovaline in whole herbage.

According to a further aspect of the present invention there is provided a combination of endophyte as described above and a host grass, wherein the combination has features selected from the group consisting of: enhancement of pest protection, resistance to insects, pasture persistence, and combinations thereof.

According to a further aspect of the present invention there is provided a combination as described above and a host grass, wherein the combination has the features of enhancement of grazing animal growth and increased animal productivity relative to grass infected with known endophytes capable of inducing

fescue toxicosis.

According to a further aspect of the present invention there is provided a combination as described above and a host grass wherein the pest to which increased resistance is conferred on the host grass is selected from the group consisting of: lesion nematode, root aphid, corn flea beetle, and combinations thereof.

According to a further aspect of the present invention there is provided seeds of a host grass infected with the endophyte substantially as described above.

According to yet a further aspect of the present invention there is provided seeds of a host grass infected with endophyte culture as described above.

The invention is the combination of examples of a class of *Neotyphodium coenophialum* endophyte and improved grass cultivars by artificial inoculation to produce host grasses which do not cause symptoms of toxicosis by way of the ergovaline toxin, but which retain sufficient levels of other alkaloids (for example: agroclavine, setoclavine and/or isosetoclavine) to individually or in combination continue to protect the host grass from pests or abiotic stresses (such as water deficit) or both.

The invention has been achieved by understanding the biology of endophytes of temperate climate grasses, isolating selected endophytes of interest, inoculating the endophytes into surface-sterilised seedlings of grasses, exemplified by improved tall fescue or perennial ryegrass cultivar lines, re-evaluating alkaloid production, multiplying seed, evaluating for agronomic factors, testing for animal production, evaluating for any evidence of animal disorders such as fescue toxicosis, staggers, hyperthermia, or prolactin hormone depression and testing for invertebrate pest protection.

The invention consists of the foregoing and also envisages constructions of which the following are examples.

BEST MODES FOR CARRYING OUT THE INVENTION

Culture conditions and description

All endophytes of this invention are strains from collections of seed of tall fescue originally sourced from the Claviplus class. Seed from various tall fescue collections were examined for the presence of endophyte by seed squash technique. A selection of plants for each seed sample, where an endophyte was shown to be present, were grown for a few weeks in glasshouse conditions and re-tested for endophyte presence in their leaf sheaths.

The endophytes from plants with chemotypes of interest were isolated and grown in culture according to the method of Latch and Christensen (1985). The endophytes of this invention are held in a culture collection or in cloned plants at the Grasslands site of AgResearch Ltd in Palmerston North, New Zealand. The cultures are also deposited at the Australian Government Analytical Laboratories in Sydney, Australia.

The accession numbers are: NM02/31935; NM02/31936; NM02/31937; NM02/31938; NM02/31939; NM02/31940; NM02/31941; NM02/31942; NM02/31943; NM02/31944; deposited on 2 October 2002. All strains of endophyte of this invention are accommodated within a single sub-grouping of the species *Neotyphodium coenophialum*. The isolates, when grown on potato dextrose agar at 22º C, are slow-growing (radial growth approximately 0.1 – 0.3 mm per day) with colonies typically white and cottony. Conidia have been observed at variable rates of production near the margin of colonies.

Inoculations

Axenic cultures of endophytes AR512, AR513, AR514, AR517, AR521, AR522, AR524, AR525, AR535, and AR539 as examples of this invention, were successfully inoculated (Latch and Christensen, 1985) into seedlings grown from surfaced sterilised seed of the tall fescue cultivar Kentucky 31. Endophytes AR514, AR524, and AR525 were also infected into other cultivars such as, for example Grasslands Flecha, Jesup Improved, Georgia 5 and various experimental lines, generally with a satisfactory success rate well in excess of 5% of attempts. No complete failures to infect tall fescue were observed with the endophytes of this invention.

A typical meadow fescue cultivar (Ensign) was successfully inoculated with endophyte AR512. Similarly a typical perennial ryegrass test line (GA66) was successfully inoculated with endophytes AR514 and AR525 for further examination with the chemotype characteristics of the combinations similar as for when infecting tall fescue, but generally with lower levels of alkaloid accumulation, and with rates of infection of the order of 1% to 5% of attempts.

Seed has been successfully produced from infected plants containing endophytes of this invention under routine seed production conditions with relatively high and useful rates of endophyte infection.

Chemotype Identification

Basal parts of endophyte-infected tillers were freeze dried, sometimes milled, and extracted and analysed qualitatively for the presence of ergovaline by high performance liquid chromatography (HPLC) as set out below. Those indicating ergovaline less than approximately 0.2 ppm of dry matter were further analysed for the production of peramine at rates greater than about 2 ppm of dry matter. The endophytes from such selections were isolated, classified by culture attributes, and generally re-inoculated into seedlings of endophyte-free tall fescue, cultivar Kentucky 31, as a typical improved pasture host for comparative purposes. Samples from such plants at various stages of growth were analysed in more detail for alkaloid production, including for the production of tremorgenic lolitrems typical of *N. lolii* infection and lolines typical of *N. coenophialum*.

The expression of alkaloid production of endophyte-infected tall fescue, using endophytes selected from tall fescue sources, was observed to fall broadly into three groups. The most numerous group produce both ergovaline and peramine at levels often well in excess of 1 ppm in the basal tiller material. Such endophytes are likely to be associated with fescue toxicosis of grazing animals if present at high rates of infection in forage.

A second chemotype group produced peramine and lolines, but no detectable trace of ergovaline. Endophytes typical of this group have been developed for commercial application (Bouton, 2000; Bouton et al., 2002, US Patent 6,111,170).

A third group, of this invention, which have low levels and a characteristic distribution within plants of ergovaline, are discussed below. They are further

characterised by the presence of peramine in herbage generally well in excess of 1 ppm, and the presence of lolines in herbage in amounts within ranges typical of *N. coenophialum* infection. In the course of chromatographic analysis for ergovaline the presence of other compounds with UV and fluorescence spectral properties typical of ergolene derivatives (i.e. fluorescent "ergot alkaloids") were observed, notably in the basal portions of tillers, crowns, and seed of tall fescue plants infected with this group of endophytes. These compounds are also discussed below.

Lolines (N-formylloline, N-acetylloline, and N-acetylnorloline, in order of usual observed abundance) were detected by capillary gas chromatography in extracts of tall fescue and meadow fescue plants infected with the endophytes of this invention in amounts more or less comparable to that observed in comparable tall fescue plants infected with common *N. coenophialum*. The methods used were minor modifications of the method of Yates et al., (1990).

Lolitrems were not detected by chromatographic analysis in any tall fescue infected with endophytes of this invention. The possible effects of lolitrems were not further directly considered, other than by observation of lambs grazing pastures containing endophyte AR514 for signs of tremors.

Ergot alkaloid levels and identification of new alkaloids

Ergovaline concentration was measured by HPLC with fluorescence detection. Typically, a sample of approximately 50 mg of milled (1 mm mesh), freeze-dried endophyte-infected tissue from the lower 3 to 5 cm of tillers from mature vegetative plants (basal tiller material predominantly consisting of leaf sheaths) was extracted with 1 ml of a mixture of equal parts of propan-2-ol and water containing also 1% lactic acid. The extraction continued for one hour at ambient temperature with gentle mixing. An internal standard of added ergotamine tartrate (c. 1 µg per sample) was used for quantitative comparisons.

Following brief centrifugation, a sample of the clarified extract solution was taken for HPLC using a reverse phase column (typically Prodigy 150 x 4.6 mm, 5µm silica ODS (3), Phenomenex, CA, USA), with elution at 1 ml per minute and a solvent gradient of acetonitrile and 100 mM aqueous ammonium acetate by volume starting at 27.5% acetonitrile and progressing in linear stages to 35% at 20 min, 50% at 35 min, 60% at 40 min and 75% at 50 min.

Naturally fluorescent ergolene compounds including ergovaline (and its isomer ergovalinine), the internal standard ergotamine (and its isomer ergotaminine partially formed during extraction) and the compounds observed in the earlier section of the chromatograms were detected by using UV excitation at 310 nm and emission at 410 nm. Ergovaline, its isomer ergovalinine, added ergotamine internal standard and its isomer ergotaminine elute at approximately 22, 36, 30 and 42 minutes respectively. The minimum detection level for routine analysis of ergovaline (combined amounts with isomer ergovalinine) is approximately 0.05 ppm of dry matter.

During the course of examining examples of tall fescue infected with endophytes of this invention for ergovaline, other ergolene derivatives were observed to be present in a pattern not previous recognised. Compounds eluting at approximately 6.5 minutes (compound A) and 8.0 minutes (compound B) are characteristically both present in endophyte-infected basal tiller and crown material of this invention. These compounds have been identified as isosetoclavine and setoclavine respectively, as follows.

Fractions enriched in compounds A and B were prepared from a methanol – 1% aqueous acetic acid (4:1) extract of bulked freeze dried and milled lower sheath of tillers containing endophytes AR514 and AR524 (pre-extracted with hexane). The extract was fractionated by sequential reverse-phase flash chromatography on octadecyl-functionalised silica gel (Aldrich) with acidic (methanol-1% aqueous acetic acid) and neutral (methanol – water) step gradients, and normal-phase flash chromatography on silica gel (silica gel 60, 40-63 μ, Merck) with an ethyl acetate – methanol step gradient.

The characteristic fluorescence and the UV absorption spectra and electrospray ionisation (ESI) mass spectral data for compounds A and B obtained by HPLC analysis of enriched fractions using variations of HPLC separation conditions and detectors (Shimadzu LC-MS instrument QP-5050 with SPD-10AVP UV diode array and RF-10A fluorescence spectral detectors), together with comparative data with standards establish these compounds are setoclavines (isosetoclavine and setoclavine respectively). Both compounds show strong fluorescence peaks in the HPLC (λ_{Ex} 310nm λ_{Em} 410nm). The UV spectra are characterised by maximum absorbances at 307 nm and 312 nm respectively and for both compounds the positive ion ESI mass spectrum shows a base peak at m/z = 237 (MH^+ - H_2O) and

an MH^+ ion at $m/z = 255$. Compound B co-eluted with a sample of reference setoclavine provided by Dr Miroslav Flieger, Institute of Microbiology Academy of Sciences of the Czech Republic. Compounds A and B were identical by HPLC, and spectral properties to isosetoclavine (compound A) and setoclavine (compound B) obtained by chemical oxidation of agroclavine by a standard procedure. The presence of isosetoclavine and setoclavine has not been previously reported in endophyte infected grasses although they have been reported as oxidation products of agroclavine in other plant systems (review by Kren, 1999).

The same extracts were also shown to contain agroclavine by electrospray LC-MS, with an ESI mass spectrum ion attributed to MH^+ at $m/z = 239$ eluting at the same time and with essentially the same UV spectrum as authentic agroclavine.

Table 1 summarises alkaloid analysis results of specified plant parts of tall fescue infected with the endophytes and usually grown under temperate summer season conditions, generally in comparative test cultivar Kentucky 31. For consideration of ergot alkaloid production in the crown and basal tiller, comparison is also made to tall fescue infected with endophytes AR501 and AR542 which consistently do not appear to produce any ergovaline or setoclavines.

Table 1. Examples and typical ranges or scores of alkaloids observed in specified plant parts

Sample (cultivar & endophyte)	Part of plant	Ergovaline (ppm DM)	Compounds A & B
Jesup EI	Whole herbage	0.4 – 1.2	ND
Jesup EI	Sheath	2.5	
Manawatu RS EI	Sheath	7.1 – 15.7	
Kentucky 31 EI	Whole Herbage	1.8 – 3.0	ND
Kentucky 31 EI	Leaf blade	0.3 – 1.8	ND
Kentucky 31 EI	Sheath	2.9 – 16.2	
Kentucky 31 AR501	Crown	< 0.1[ND]	*
Kentucky 31 AR514	Whole herbage	< 0.1	*
Kentucky 31 AR514	Crown	0.1 – 0.6	**
Kentucky 31 AR514	Immature heads	< 0.1 – 0.2	*
Kentucky 31 AR522	Stem & sheath	0.1 – 0.2	*
Kentucky 31 AR522	Crown	3.4 – 6.1	***
Kentucky 31 AR524	Crown	0.3 – 0.6	**
Kentucky 31 AR524	Immature heads	< 0.1	*
Kentucky 31 AR525	Whole herbage	< 0.1	ND
Kentucky 31 AR525	Crown	0.7 – 1.0	***
Kentucky 31 AR525	Immature heads	< 0.1	*
Kentucky 31 AR525	Stem, sheath & heads	<0.1	*
Kentucky 31 AR535	Crown	0.4 – 0.7	***
Kentucky 31 AR535	Stem, sheath & heads	<0.1	**
Kentucky 31 AR542	Crown	< 0.1	ND
Kentucky 31 AR542	Sheath	< 0.1	ND

Compound A = isosetoclavine

Compound B = setoclavine

ND = not detected, detection limit of 0.1 ppm DM for ergovaline

* = possible trace or low level

** and *** = score of relative abundance observed

EI = infected with common toxic or wild type endophyte

Genotype characterisation of endophyte

All endophytes discussed above are characterised by DNA "fingerprinting" (selected polymorphic microsatellite loci and/or Arbitrary Fragment Length Polymorphisms (AFLP) technique) as belonging to a sub-group of *Neotyphodium coenophialum*.

Samples of about 50 mg fresh or 15 mg dry basal tiller were used for the extraction of DNA using FastDNA kit for plants (Bio 101,Vista, California) by procedures recommended with the kit. Alternatively genomic DNA was extracted from cultured endophyte (Moon et al., 1999). Microsatellite PCR amplification was performed using primer pairs labelled with fluorescent dyes, B10.1 (5'-TET) / B10.2 and B11.1 (5'-HEX) / B11.4, as described by Moon et al., (1999). The apparent size of microsatellite PCR fluorescent labelled products was measured relatively to within an estimated 0.3 nucleotide units by capillary electrophoresis using an ABI 3100 Genetic Analyzer with POP6 polymer chemistry in 50 cm capillary arrays and GeneScan-400HD standards (Applied Biosystems Inc., Foster City, CA). The apparent sizes of PCR products by this technique (adjusted by subtracting a unit where an adenine nucleotide appears to have been terminally added) are set out in Table 2.

Table 2 shows that the endophytes of this invention can be distinguished from other groups of *Neotyphodium* endophytes by the number of alleles observed and the apparent sizes of such alleles. Thus all strains of this invention share a B11 allele of size c. 128 base pairs and a second B11 allele within the size range c. 192 to 200bp. They also share with other *N. coenophialum* strains up to three B10 alleles within the range c.154 to 185bp.

The presence of three alleles for the endophytes of this invention for the B10 locus is consistent with evidence for *N. coenophialum* as a hybrid endophyte derived from three different *Epichloë* source species (Tsai et al., 1994).

Table 2. Apparent size of B10 and B11 microsatellite PCR products.

	B10 locus		B11 locus	
Source material	No of alleles	Allele sizes (bp)	No of alleles	Allele sizes (bp)
N. coenophialum, wild type, Australian C1, coB isozymes	3	160.4, 169.6, 184.2	2	147.9, 192.2
N. coenophialum, wild type, NZ Tindall's, coB isozymes	2	160.4, 169.4	2	147.9, 192.2
N. coenophialum, wild type, NZ RS2 & NZ RS6	3	160.3, 169.3, 184.2	2	147.8, 192.0
N. coenophialum, AR542	2	160.5, 169.6	2	180.4, 192.2
Neotyphodium sp., FaTG-3 strain AR501	2	169.5, 178.7	1	127.9
Neotyphodium sp., FaTG-3 strain AR506	2	169.6, 178.7	1	127.8
AR539	3	154.6, 172.5, 178.3	2	127.9, 192.2
AR513	3	157.7, 160.5, 178.4	2	128.0, 192.2
AR525	3	157.7, 160.4, 178.3	2	128.0, 192.2
AR517	3	163.4, 172.5, 178.2	2	128.0, 192.1
AR521	3	163.3, 172.5, 178.2	2	127.9, 192.1
AR512	3	172.6, 178.5, 181.5	2	128.0, 192.2
AR514	3	157.8, 160.6, 178.4	2	128.0, 196.2
AR522	3	157.7, 160.5, 178.3	2	128.0, 200.1
AR524	3	157.7, 160.5, 178.3	2	127.9, 200.2
AR535	3	157.7, 160.5, 178.3	2	128.0, 200.1

Analysis by AFLP (Griffiths et al., 1999) also confirmed that endophyte examples AR514, AR525 and AR535 of this invention are from a sub-group which can be distinguished from other *N. coenophialum* endophytes outside this sub-group by one or more polymorphic differences, but not many differences, from within more than 200 AFLP bands observed to be polymorphic for the genera *Neotyphodium* and *Epichloë*.

Safe grazing with endophyte in tall fescue cultivar Kentucky 31

Pastures of tall fescue infected with examples of the endophytes of this invention do not induce typical fescue toxicosis in grazing animals. Table 3 shows growth rate of lambs in a trial conducted at Eatonton, GA, USA for two seasons (21 April –

30 June 1997, 2 April – 2 July 1998). The growth of lambs on a pasture of Kentucky 31 tall fescue infected with endophyte strain AR514 was essentially the same growth as on equivalent endophyte-free pasture and significantly better than growth on naturally endophyte-infected pasture using the comparable Jesup cultivar. The wild type endophyte infection significantly reduced live weight gain (P<0.05) and increased mean body (rectal) temperature.

Gross depression of prolactin in blood is another symptom of fescue toxicosis. Endophyte strain AR514 did not cause a depression of prolactin whereas with the wild type endophyte prolactin was grossly reduced.

Overall, the performance of lambs grazing on AR514 pasture was similar to that on the endophyte-free pasture. No tremors or “ryegrass staggers” symptoms were observed.

Table 3. Performance of lambs grazing endophyte infected and endophyte free pasture.

		Endophyte treatment		
	Year	AR514 (in Kentucky 31)	Jesup EF*	Jesup EI*
Live weight gain (g/hd/d)	1997	103 a**	102 a	67 b
	1998	93 a	102 a	57 b
Body temperature (ºC)	1997	39.8 a	39.8 a	40.1 b
Blood prolactin (ng/ml)	1997	414 a	400 a	Not detectable (b)
	1998	550 a	150 a	< 0.5 b

* EF = endophyte free; EI = infected with common toxic or wild type endophyte.

** Treatments with no letter in common are significantly different (P<0.05).

Endophytes and resistance to lesion nematodes

In some environments, notably sandy soils with relatively warm and humid climates, nematode may cause significant damage to tall fescue root systems thus affecting the persistence of the grass in the pasture. A greenhouse trial with three plants and nine replicates per treatment has demonstrated that endophyte infection may confer resistance to lesion nematodes, *Pratylenchus* spp. In a greenhouse

experiment nematode reproduction was investigated with two tall fescue cultivars infected with various endophytes or endophyte free.

Table 4 indicates that an endophyte of this invention, AR514, confers partial resistance to lesion nematodes to a greater degree than endophyte-free plants (EF) or two endophytes lacking production of ergovaline (AR542 & AR584) although not to the same degree as the common endophyte (EI) of the cultivars.

Table 4. Endophyte effect on lesion nematode numbers

Fescue cultivar	**Endophyte**				
	EF*	AR542	AR584	AR514	EI*
GA 5	146	149	101	-	19
Jesup	147	88	120	69	30
Mean	146 a **	118 ab	111 ab	69 b	24 c
Relative to EF= 100	*100 a*	*81 ab*	*76 ab*	*47 b*	*16 c*

* EF = endophyte free; EI = infected with common toxic or wild type endophyte.

** Treatments with no letter in common are significantly different (P<0.05).

Endophytes confer resistance to root aphid

Endophyte infection is known to affect infestation of grasses by aphids. Table 5 compares treatments of fourteen plants each of tall fescue cultivar Kentucky 31 for mean numbers of root aphids wherein AR514 infection is shown to confer considerable protection in comparison with another endophyte-infected set of plants or endophyte free plants.

Table 5. Log number of root aphid per 10 ml sub-sample in Kentucky 31 tall fescue.

Endophyte treatment	**No. root aphid**	**No. root aphid/gm root**
Endophyte Free	4.043 a*	2.055 a
AR542	1.710 b	0.473 b
AR514	0.765 c	0.095 c

* Treatments with no letter in common are significantly different (P<0.01)

Endophytes confer deterrence to corn flea beetle

Tall fescue Kentucky 31 leaves infected with examples of the endophytes of this invention (E+) and also leaves infected with wild type toxic endophyte were compared with endophyte free (EF) material using the corn flea beetle *Chaetocnema pulicaria* in a feeding preference test experiment. Endophytes AR512, AR513, AR514, AR524 and AR525 all conferred resistance or feeding deterrence similar to that of leaves infected with the wild type toxic endophyte. The mean of feeding scores for the examples of this invention were E+ = 0.4 as against EF = 2.8 ($P<0.001$) on a scale of 0 to 3 (where 0 is no feeding and 3 is extensive feeding). Score counts of feeding scars (E+ = 2.2, EF = 27.8, mean of the total number of scars across 3 transects per leaf blade) and of faecal pellets (E+ = 9.2, EF = 75.8, mean number of faecal pellets on each blade) were also highly significantly different with those for E+ closely comparable to those of toxic wild type endophyte of Kentucky 31.

A similar single preference test with endophyte AR512 infected into meadow fescue cultivar Ensign demonstrated an even more extreme preference effect with feeding scores of E+ = 0.0, EF = 3.0, feeding scars score counts E+ = 0, EF = 33, and faecal pellets E+ = 3, EF = 50.

Aspects of the present invention have been described by way of example only and it should be appreciated that modifications and additions may be made thereto without departing from the scope thereof.

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WHAT WE CLAIM IS:

1. An endophyte of *Neotyphodium coenophialum* species, selected from the group consisting of: AR512; AR513; AR514; AR517; AR521; AR522; AR524; AR525; AR535; AR539; and combinations thereof; AR512; AR513; AR514; AR517; AR521; AR522; AR524; AR525; AR535, AR539 being cultures deposited on 2 October 2002 at the Australian Government Analytical Laboratories (AGAL) with accession numbers: NM02/31935; NM02/31936; NM02/31937; NM02/31938; NM02/31939; NM02/31940; NM02/31941; NM02/31942; NM02/31943; NM02/31944;

characterised in that, in combination with a host grass, said endophyte does not cause symptoms of toxicosis in animals;

and further characterised in that the endophyte retains sufficient levels of at least two alkaloids selected from the group consisting of: agroclavine; setoclavine; isosetoclavine; and combinations thereof, that protect the host grass from pests or abiotic stresses or both;

and further characterised in that the host grass is artificially inoculated with the endophyte.

2. An endophyte as claimed in claim 1, characterised in that the toxicosis is fescue toxicosis.

3. An endophyte as claimed in claim 1 or claim 2, characterised in that the toxicosis is caused by ergovaline.

4. An endophyte as claimed in claim 2 or claim 3 characterised in that the level of ergovaline is less than 0.4 ppm in dry matter in herbage consumed by grazing animals.

5. An endophyte as claimed in either claim 3 or 4, characterised in that the level of ergovaline is less than 0.4 ppm in dry matter in herbage, other than the crown of the host grass plant, consumed by grazing animals.

6. An endophyte as claimed in any one of the preceding claims characterised

in that the abiotic stress is a water deficit.

7. An endophyte culture of *Neotyphodium coenophialum* species, selected from the group consisting of: AR512; AR513; AR514; AR517; AR521; AR522; AR524; AR525; AR535; AR539; and combinations thereof; AR512; AR513; AR514; AR517; AR521; AR522; AR524; AR525; AR535, AR539 being cultures deposited on 2 October 2002 at the Australian Government Analytical Laboratories (AGAL) with accession numbers: NM02/31935; NM02/31936; NM02/31937; NM02/31938; NM02/31939; NM02/31940; NM02/31941; NM02/31942; NM02/31943; NM02/31944;

characterised in that, in combination with a host grass, said endophyte culture does not cause symptoms of toxicosis in grazing animals;

and further characterised in that said endophyte culture retains sufficient levels of at least two alkaloids selected from the group consisting of: agroclavine; setoclavine; isosetoclavine; and combinations thereof, that protect the host grass from pests or abiotic stresses or both;

and further characterised in that the host grass is artificially inoculated with the endophyte culture.

8. An endophyte culture as claimed in claim 7 characterised in that the endophyte culture is an axenic culture.

9. An endophyte culture as claimed in claim 7 or claim 8, characterised in that the toxicosis is fescue toxicosis.

10. An endophyte culture as claimed in any one of claim 7 to 9, characterised in that the toxicosis is caused by ergovaline.

11. An endophyte culture as claimed in claim 9 or claim 10 characterised in that the level of ergovaline is less than 0.4 ppm in dry matter in herbage consumed by grazing animals.

12. An endophyte culture as claimed in any one of claims 9 to 11, characterised in that the level of ergovaline is less than 0.4 ppm in dry matter in herbage, other than the crown of the host grass plant, consumed by grazing animals.

13. An endophyte culture as claimed in any one of claims 7 to 12 characterised in that the abiotic stress is a water deficit.

14. A combination of an endophyte as claimed in any one of claims 1 to 6, and a host grass.

15. A combination of an endophyte culture as claimed in any one of claims 7 to 13, and a host grass.

16. A combination as claimed in either claim 14 or 15 characterised in that said combination is achieved by modification of host grass infected with the endophyte or endophyte culture by methods selected from the group consisting of: breeding; crossing; hybridisation; genetic modification; and combinations thereof..

17. A combination as claimed in any one of claims 14 to 16 characterised in that said host grass is selected from the group consisting of: tall fescue grass cultivar; ryegrass cultivar; meadow fescue cultivar; and combinations thereof.

18. A combination as claimed in any one of claims 14 to 16 characterised in that said host grass is a Pooideae grass.

19. A combination as claimed in any one of claims 14 to 18 characterised in that the combination produces isosetoclavine and setoclavine at a rate of greater than 0.5 ppm each of dry matter in the host grass plant crowns

20. A combination as claimed in any one of claims 14 to 19 characterised in that the combination produces less than 0.2 ppm of dry matter of ergovaline in whole herbage.

21. A combination as claimed in any one claims 14 to 20 characterised in that the combination has at least one feature selected from the group consisting of: enhancement of pest protection; resistance to insects; pasture persistence; and a combination thereof.

22. A combination as claimed in any one of claims 14 to 21, characterised in that the combination has the features of enhancement of grazing animal growth and/or increased animal productivity relative to grass infected with known endophytes capable of inducing fescue toxicosis.

23. A combination as claimed in any one of claims 14 to 22 characterised in that the pest, to which increased resistance is conferred on the host grass, is selected from the group consisting of: lesion nematode; root aphid; corn flea beetle; and combinations thereof.

24. Seed of a host grass characterised in that the seeds are from a host grass infected with endophyte as claimed in any one of claims 1 to 6.

25. Seed of a host grass characterised in that the seeds are from a grass infected with an endophyte culture as claimed in any one of claims 7 to 13.

26. An endophyte as claimed in any one of claims 1 to 6 and substantially as hereinbefore described with reference to the accompanying examples.

27. An endophyte culture as claimed in any one of claims 7 to 13 and substantially as hereinbefore described with reference to the accompanying examples.

28. A combination as claimed in any one of claims 14 to 23 and substantially as hereinbefore described with reference to the accompanying examples.

29. Seed as claimed in either claims 24 or 25 and substantially as hereinbefore described with reference to the accompanying examples.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NZ03/00219

A.	**CLASSIFICATION OF SUBJECT MATTER**
Int. Cl. [7]:	C12N 1/14, A01N 63/04
According to International Patent Classification (IPC) or to both national classification and IPC	
B.	**FIELDS SEARCHED**
Minimum documentation searched (classification system followed by classification symbols) SEE ELECTRONIC DATABASE BOX BELOW	
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched SEE ELECTRONIC DATABASE BOX BELOW	
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) CA, CAPLUS, MEDLINE, BIOSIS, WPIDS, AGRICOLA: keywords: Neotyphdium, coenphialum, acremonium, toxicosis	

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 6111170, A (Latch G C M *et al.*) 29 August 2000. See whole document.	1-29
P, X	Parish J A *et al.* (2003) J. Anim. Sci. 81: 1316-1322. "Use of nonergot alkaloid-producing endophytes for alleviating tall fescue toxicosis in sheep". See whole document.	1-29
A	WO 00/40075, A (Advanta Seeds Pacific) 13 July 2000. See whole document.	

[X] Further documents are listed in the continuation of Box C   [X] See patent family annex

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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Date of the actual completion of the international search 12 December 2003	Date of mailing of the international search report 19 DEC 2003
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaustralia.gov.au Facsimile No. (02) 6285 3929	Authorized officer JANE MCHENRY Telephone No : (02) 6283 2091

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages *(Remove spaces when completed if the page is too long)*	Relevant to claim No.
A	WO 02/13616, A (The Board of Trustees of the University of Arkansas) 21 February 2002. See whole document.	
A	JP 2002-209441 (Japan Grassland Farming Forage Seed Association) 30 July 2002. See whole document.	
A	WO 00/62600, A (Advanta Seeds Pacific Inc.) 26 October 2000. See whole document.	
A	Roberts C A *et al.* (2002) J. Agric. Food. Chem. 50: 5742-5745. "Use of rat model to evaluate tall fescue seed infected with introduced strains of Neotyphodium coenophialum" See whole document.	
A	Joost R E (1995) J Anim. Sci. 73: 881-888. "Acremonium in Fescue and Ryegrass: Boon or Bane? A review". See whole document.	
A	Schardl C L *et al.* (2001) Phytopathology 91(6): S123. "The gene for the determinant step in ergot alkaloid synthesis by Neotyphodium coenophialum and other grass endophytes" See whole document.	
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International application No.
PCT/NZ03/00219

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member					
US	6111170	CA	2319847				
WO	0062600	AU	24886/00	AU	42422/00	WO	0040075
WO	0213616	AU	84955/01	US	2003064055		
JP	2002-209441						

END OF ANNEX

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